



## Association of a polymorphism of the apolipoprotein E gene with chronic kidney disease in Japanese individuals with metabolic syndrome

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### ABSTRACT

The purpose of the present study was to identify genetic variants that confer susceptibility to chronic kidney disease (CKD) in Japanese individuals with metabolic syndrome. The study population comprised 2150 Japanese individuals with metabolic syndrome, including 411 subjects with CKD [estimated glomerular filtration rate (eGFR) <50 mL/min/1.73m<sup>2</sup>] and 1739 controls (eGFR ≥60 mL/min/1.73m<sup>2</sup>). The genotypes for 100 polymorphisms of 80 candidate genes were determined. The chi-square test, multivariable logistic regression analysis with adjustment for covariates, as well as a stepwise forward selection procedure revealed that nine polymorphisms of APOE, ABCA1, PTGS1, TNF, CPB2, AGTR1, OR13G1, and GNB3 were associated ( $P < 0.05$ ) with the prevalence of CKD. Among these polymorphisms, the -219G → T polymorphism of APOE (rs405509) was most significantly associated with CKD in Japanese individuals with metabolic syndrome.

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### Introduction

Chronic kidney disease (CKD) is a global public health problem [1], being the leading cause of end-stage renal disease (ESRD) as well as a major determinant of poor cardiovascular outcome and premature death [2,3]. Identification of genetic markers for CKD risk is thus important both for risk prediction and for intervention to avert future ESRD and cardiovascular events.

Metabolic syndrome is defined by a clustering of abdominal obesity, a high serum concentration of triglycerides, a low serum concentration of high density lipoprotein (HDL)-cholesterol, high blood pressure, and an increased fasting blood glucose level [4]. It has been recognized as an important risk factor for CKD [5–7] as well as for coronary heart disease [8,9] and ischemic stroke [10,11]. Genetic factors underlying predisposition to CKD in individuals with metabolic syndrome have remained largely unknown, however. Furthermore, given the ethnic differences in lifestyle and environmental factors as

well as in genetic background, it is important to examine genetic variants related to CKD in individuals with metabolic syndrome in each ethnic group.

We have now performed an association study for 100 polymorphisms of 80 candidate genes and CKD in 2150 Japanese individuals with metabolic syndrome. The purpose of the present study was to identify genetic variants that confer susceptibility to CKD among individuals with metabolic syndrome and thereby to provide a basis for the personalized prevention of this condition.

### Results

The characteristics of the 2150 study subjects are shown in Table 1. Age, the prevalence of hypertension, diabetes mellitus, myocardial infarction, and ischemic stroke, as well as systolic blood pressure were greater, whereas BMI and the percentage of smokers were lower, in subjects with CKD than in controls.

Comparison of allele frequencies with the chi-square test revealed that 14 polymorphisms were related ( $P$  value of <0.05 for allele frequency) to the prevalence of CKD (Table 2). Among these

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**Table 1**

Characteristics of individuals with chronic kidney disease (CKD) and controls among subjects with metabolic syndrome

Characteristic	CKD	Controls	P
No. of subjects	411	1739	
Age (years)	70.5±9.2	66.0±9.5	<0.0001
Sex (female/male, %)	37.7/62.3	39.6/60.4	0.4755
Body mass index (kg/m <sup>2</sup> )	24.6±3.6	25.2±5.8	0.0033
Current or former smoker (%)	18.5	28.6	<0.0001
Hypertension (%)	86.1	73.7	<0.0001
Systolic blood pressure (mmHg)	153±26	145±22	<0.0001
Diastolic blood pressure (mmHg)	82±15	82±14	0.6625
Hypercholesterolemia (%)	34.8	33.8	0.7062
Serum total cholesterol (mmol/L)	5.31±1.13	5.26±1.01	0.4738
Serum triglyceride (mmol/L)	2.18±1.16	2.14±1.38	0.6102
Serum HDL-cholesterol (mmol/L)	1.16±0.39	1.20±0.31	0.0805
Diabetes mellitus (%)	56.5	40.4	<0.0001
Fasting plasma glucose (mmol/L)	7.75±3.31	7.54±3.16	0.3451
Glycosylated hemoglobin (%)	6.37±1.66	6.15±1.64	0.1085
Serum creatinine (μmol/L)	146.9±167.2	62.8±12.4	<0.0001
eGFR (mL min <sup>-1</sup> 1.73 m <sup>-2</sup> )	39.3±10.5	78.7±15.4	<0.0001
End-stage renal disease (%)	5.1	0	<0.0001
Myocardial infarction (%)	34.1	25.7	0.0008
Ischemic stroke (%)	20.4	12.8	0.0001

Quantitative data are means±SD. Smoker: smoking of ≥10 cigarettes daily. Hypertension: systolic blood pressure of ≥140 mmHg, diastolic blood pressure of ≥90 mmHg, or taking antihypertensive medication. Hypercholesterolemia: serum total cholesterol of ≥5.72 mmol/L (220 mg/dL) or taking lipid-lowering medication. Diabetes mellitus: fasting blood glucose of ≥6.93 mmol/L (126 mg/dL), glycosylated hemoglobin (hemoglobin A1c) content of ≥6.5%, or taking antidiabetes medication. eGFR, estimated glomerular filtration rate.

polymorphisms, the -219G→T polymorphism of *APOE* was significantly [false discovery rate (FDR)<0.05] associated with this condition. The genotype distributions of all 14 polymorphisms (Table 2), with the exception of the T→C (Ile347Thr) polymorphism of *CPB2* in control subjects, were in Hardy-Weinberg equilibrium both in individuals with CKD and in controls (Supplementary Table 1); the T→C (Ile347Thr) polymorphism of *CPB2* was therefore excluded from subsequent analysis.

Multivariable logistic regression analysis with adjustment for age, sex, and the prevalence of hypertension, diabetes mellitus, and hypercholesterolemia revealed that the -219G→T polymorphism of *APOE* (dominant, recessive, and additive 2 models), the G→A (Ala163Thr) polymorphism of *AGTR1* (dominant and additive 1 models), the C→T polymorphism of *PTGS1* (recessive and additive 2 models), the -863C→A polymorphism of *TNF* (dominant and additive 1 models), the G→A (Ala147Thr) polymorphism of *CPB2* (dominant model), the 2583A→G (Ile823Met) (recessive and additive 2 models) and -14C→T (recessive and additive 2 models) polymorphisms of *ABCA1*, the 1429C→T polymorphism of *GNB3* (recessive and additive 2 models), and the A→G (Ile132Val) polymorphism of *OR13G1* (dominant and additive 2 models) were significantly ( $P<0.05$ ) associated with the prevalence of CKD (Table 3). The variant *T* allele of *APOE*, *A* allele of *AGTR1*, *T* allele of *PTGS1*, *T* allele of the -14C→T polymorphism of *ABCA1*, and *G* allele of *OR13G1* were risk factors for CKD, whereas the variant *A* allele of *TNF*, *A* allele of *CPB2*, *G* allele of the 2583A→G (Ile823Met) polymorphism of *ABCA1*, and *T* allele of *GNB3* were protective against this condition.

Finally, we performed a stepwise forward selection procedure to examine the effects of genotypes for the nine polymorphisms associated with CKD by multiple logistic regression analysis as well as of age, sex, and the prevalence of hypertension, diabetes mellitus, and hypercholesterolemia on CKD (Table 4). Age, diabetes mellitus, hypertension, *APOE* genotype (recessive model), *ABCA1* genotype (the -14C→T polymorphism, recessive model), *PTGS1* genotype (recessive model), *TNF* genotype (dominant model), *CPB2* genotype (dominant model), *AGTR1* genotype (dominant model), *OR13G1* genotype (dominant model), *GNB3* genotype (recessive model), and *ABCA1*

genotype [the 2583A→G (Ile823Met) polymorphism, recessive model], in descending order of statistical significance, were significant ( $P<0.05$ ) and independent determinants of CKD.

## Discussion

We examined the possible relations of 100 polymorphisms in 80 candidate genes to the prevalence of CKD in 2150 Japanese individuals with metabolic syndrome. Our results show that the -219G→T polymorphism of *APOE* was significantly associated with CKD in such individuals, with the variant *T* allele representing a risk factor for this condition.

Apolipoprotein E (*APOE*) is a structural component of both chylomicrons and very low density lipoprotein remnants, and it is responsible for the binding and uptake of these particles by the low density lipoprotein (LDL) receptor and LDL receptor-like protein [12,13]. Three common alleles ( $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$ ) of *APOE* encode the three major isoforms (E2, E3, and E4) of *APOE*, which differ at amino acid positions 112 and 158. Allelic variation of *APOE* accounts for interindividual variability in total cholesterol and LDL-cholesterol concentrations, with studies in human populations having demonstrated associations of the  $\epsilon 4$  and  $\epsilon 2$  alleles with increased and decreased LDL-cholesterol levels, respectively [14–16]. The relation of *APOE* polymorphisms to coronary heart disease or myocardial infarction has been extensively investigated, with the  $\epsilon 4$  allele having been associated with these conditions in many studies [17–19]. A meta-analysis of 15,492 subjects with coronary heart disease and 32,965 controls pooled from 48 studies revealed that, compared with individuals with the  $\epsilon 3/\epsilon 3$  genotype, carriers of the  $\epsilon 4$  allele had a higher risk for coronary heart disease (odds ratio, 1.42), whereas the  $\epsilon 2$  allele was not associated with risk for this condition [20]. The  $\epsilon 4$  allele of *APOE* is thus an important risk factor for coronary heart disease. In a prospective cohort study of 3859 African American and 10,661 white adults with a median follow-up time of 14 years, the  $\epsilon 2$  allele moderately increased and the  $\epsilon 4$  allele reduced the risk of CKD progression [21]. This study suggested that *APOE* variation predicts CKD progression independently of diabetes, race, as well as lipid and nonlipid risk factors, and that nonlipid pathways, such as cellular mechanisms of kidney remodeling, may contribute to the association of *APOE* alleles with progression of CKD [21]. In Japanese cohorts, the  $\epsilon 4$  allele of *APOE* was also protective against the progression of diabetic nephropathy [22], whereas the  $\epsilon 2$  allele was associated with an increased prevalence of ESRD [23]. These various observations implicate *APOE* as a candidate susceptibility gene for CKD as well as coronary heart disease, although the risk alleles differ between these conditions.

The -219G→T polymorphism of *APOE* was previously associated with myocardial infarction in men in France and Northern Ireland, with the *T* allele representing a risk factor for this condition [24]. The *T* allele of this polymorphism was also shown to be a risk factor for coronary heart disease in low-risk Japanese men [25]. We have now shown that the -219G→T polymorphism of *APOE* was significantly associated with the prevalence of CKD in individuals with metabolic syndrome, with the *T* allele representing a risk factor for CKD. Consistent with its location in the promoter region of *APOE*, the -219G→T polymorphism was previously shown to be associated with the plasma concentration of *APOE*, with the *T* allele conferring a reduced *APOE* concentration, but this polymorphism was found not to be related to plasma lipid profile [24]. The deleterious influence of the *T* allele on coronary heart disease and CKD therefore cannot be explained by its effect on the circulating level of *APOE*. Although an abnormality in lipoprotein metabolism may play an important role in the acceleration of atherosclerosis and the development of global glomerulosclerosis [26,27], the mechanism underlying the association of the -219G→T polymorphism of *APOE* with CKD remains to be determined.

**Table 2**Genotype distributions of polymorphisms related ( $P < 0.05$  for allele frequency) to chronic kidney disease (CKD) by the chi-square test among individuals with metabolic syndrome

Gene symbol	Polymorphism	dbSNP	CKD	Controls	<i>P</i> (genotype)	<i>P</i> (allele frequency)	FDR
APOE	–219G→T	rs405509			0.0016	0.0004	0.043
	GG		23 (5.6)	148 (8.5)			
	GT		149 (36.3)	744 (42.8)			
	TT		239 (58.1)	847 (48.7)			
AGTR1	G→A (Ala163Thr)	rs12721226			0.0044	0.0045	0.223
	GG		408 (99.3)	1738 (99.9)			
	GA		3 (0.7)	1 (0.1)			
	AA		0 (0)	0 (0)			
PTGS1	C→T	rs883484			0.0349	0.0141	0.276
	CC		143 (34.8)	684 (39.3)			
	CT		191 (46.5)	811 (46.7)			
	TT		77 (18.7)	244 (14.0)			
TNF	–863C→A	rs1800630			0.0588	0.0194	0.276
	CC		320 (77.9)	1254 (72.1)			
	CA		85 (20.7)	449 (25.8)			
	AA		6 (1.4)	36 (2.1)			
CPB2	G→A (Ala147Thr)	rs3742264			0.0666	0.0196	0.276
	GG		249 (60.6)	957 (55.0)			
	GA		142 (34.6)	657 (37.8)			
	AA		20 (4.9)	125 (7.2)			
UCP2	–866G→A	rs659366			0.0605	0.0214	0.276
	GG		97 (23.6)	479 (27.5)			
	GA		204 (49.6)	881 (50.7)			
	AA		110 (26.8)	379 (21.8)			
LRP8	T→G (Asp46Glu)	rs3820198			0.0547	0.0261	0.276
	TT		130 (31.6)	492 (28.3)			
	TG		210 (51.1)	855 (49.2)			
	GG		71 (17.3)	392 (22.5)			
ABCA1	2583A→G (Ile823Met)	rs4149313			0.0886	0.0275	0.276
	AA		68 (16.5)	242 (13.9)			
	AG		195 (47.5)	773 (44.5)			
	GG		148 (36.0)	724 (41.6)			
ABCA1	–14C→T	rs1800977			0.0306	0.0301	0.276
	CC		217 (52.8)	985 (56.7)			
	CT		156 (38.0)	652 (37.5)			
	TT		38 (9.2)	101 (5.8)			
GNB3	1429C→T	rs5446			0.0699	0.0311	0.276
	CC		287 (69.8)	1136 (65.4)			
	CT		115 (28.0)	528 (30.4)			
	TT		9 (2.2)	74 (4.2)			
OR13G1	A→G (Ile132Val)	rs1151640			0.0660	0.0344	0.276
	AA		266 (15.3)	266 (15.3)			
	AG		782 (45.0)	782 (45.0)			
	GG		691 (39.7)	691 (39.7)			
SERPINE1	A→G (Tyr243Cys)	rs13306846			0.0361	0.0361	0.276
	AA		409 (99.5)	1738 (99.9)			
	AG		2 (0.5)	1 (0.1)			
	GG		0 (0)	0 (0)			
CPB2	T→C (Ile347Thr)	rs1926447			0.0223	0.0442	0.276
	TT		9 (2.2)	46 (2.7)			
	TC		122 (29.7)	404 (23.2)			
	CC		280 (88.1)	1289 (74.1)			
APOA5	–3A→G	rs651821			0.1433	0.0488	0.276
	AA		170 (41.4)	636 (36.6)			
	AG		187 (45.5)	827 (47.6)			
	GG		54 (13.1)	275 (15.8)			

We found that eight polymorphisms of *ABCA1*, *PTGS1*, *TNF*, *CPB2*, *AGTR1*, *OR13G1*, and *GNB3* were also related to the prevalence of CKD in individuals with metabolic syndrome, with none of these polymorphisms having previously been shown to be associated with CKD. The –14C→T and 2583A→G (Ile823Met) polymorphisms of *ABCA1* have been shown to affect the plasma level of HDL-cholesterol in Japanese [28] and Turkish [29] populations. The T allele of the 1429C→T polymorphism of *GNB3* has also been shown to be a risk factor for the development of hypercholesterolemia in Japanese males [30]. The C→T polymorphism of *PTGS1* [31], the G→A (Ala147Thr) polymorphism of *CPB2* [32], and the A→G (Ile132Val) polymorphism of *OR13G1* [33] were previously found to be associated with myocardial infarction or coronary heart disease. Although the 1166A→C polymorphism of *AGTR1* [34,35] and the –308G→A

polymorphism of *TNF* [36,37] were shown to be associated with CKD, the G→A (Ala163Thr) polymorphism of *AGTR1* and the –863C→A polymorphism of *TNF* have not previously been associated with this condition in a prospective cohort or case-control study. The A allele of the –863C→A polymorphism of *TNF* was shown to be protective against the development of coronary artery disease in Japanese men without type 2 diabetes [38].

Our study has several limitations: (1) We used eGFR instead of directly measured GFR to define CKD. (2) We were not able to obtain information about the underlying renal disease in all subjects with CKD. Such information can be obtained by detailed clinical examination, including renal biopsy, but such diagnostic procedures are not considered feasible for a study whose subjects are recruited from the general population. (3) It is possible that one or more of the

**Table 3**

Multivariable logistic regression analysis of polymorphisms related to chronic kidney disease for individuals with metabolic syndrome

Gene symbol	Polymorphism	Dominant		Recessive		Additive 1		Additive 2	
		P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)
APOE	-219G→T	<b>0.0234</b>	1.72 (1.10–2.80)	<b>0.0006</b>	1.48 (1.18–1.86)	0.1675		<b>0.0049</b>	1.98 (1.25–3.27)
AGTR1	G→A (Ala163Thr)	<b>0.0275</b>	13.3 (1.6–271.7)			<b>0.0275</b>	13.3 (1.6–271.7)		
PTGS1	C→T	0.1805		<b>0.0073</b>	1.50 (1.11–2.00)	0.6198		<b>0.0084</b>	1.55 (1.12–2.14)
TNF	-863C→A	<b>0.0150</b>	0.72 (0.55–0.93)	0.2508		<b>0.0268</b>	0.74 (0.56–0.96)	0.1920	
CPB2	G→A (Ala147Thr)	<b>0.0236</b>	0.77 (0.61–0.96)	0.1003		0.0656	0.0510		
UCP2	-866G→A	0.1797		0.1016		0.3890		0.0679	
LRP8	T→G (Asp46Glu)	0.3999		0.0512		0.8432		0.0714	
ABCA1	2583A→G (Ile823Met)	0.0628		<b>0.0181</b>	0.76 (0.60–0.95)	0.2688		<b>0.0148</b>	0.66 (0.47–0.92)
ABCA1	-14C→T	0.1149		<b>0.0047</b>	1.79 (1.18–2.67)	0.4366		<b>0.0034</b>	1.86 (1.22–2.80)
GNB3	1429C→T	0.2074		<b>0.0407</b>	0.47 (0.22–0.92)	0.4960		<b>0.0352</b>	0.46 (0.21–0.90)
OR13G1	A→G (Ile132Val)	<b>0.0308</b>	1.47 (1.05–2.10)	0.2748		0.0532		<b>0.0306</b>	1.50 (1.05–2.19)
SERPINE1	A→G (Tyr243Cys)	0.0923		0.2417		0.2362		0.0923	
APOA5	-3A→G	0.1443		0.2995		0.2392		0.1598	

OR, odds ratio; CI, confidence interval. Multivariable logistic regression analysis was performed with adjustment for age, sex, and the prevalence of hypertension, diabetes mellitus, and hypercholesterolemia. *P* values of <0.05 are shown in bold.

polymorphisms associated with CKD in the present study are in linkage disequilibrium with other polymorphisms in the same gene or in other nearby genes that are actually responsible for the development of this condition. (4) The functional relevance of the identified polymorphisms to gene transcription or to protein structure or function was not determined in the present study.

In conclusion, our present results suggest that the -219G→T polymorphism of APOE is significantly associated with CKD in Japanese individuals with metabolic syndrome. Validation of our findings will require their replication with independent subject panels.

## Materials and methods

### Study population

The study population comprised 2150 unrelated Japanese individuals (1306 men, 844 women) who either visited outpatient clinics or were admitted to one of the participating hospitals (Gifu Prefectural General Medical Center and Gifu Prefectural Tajimi Hospital in Gifu Prefecture, Japan; and Hirosaki University Hospital, Reimeikyo Rehabilitation Hospital, and Hirosaki Stroke Center in Aomori Prefecture, Japan) between October 2002 and March 2008 because of various symptoms or for an annual health checkup, or who were recruited to a population-based prospective cohort study of aging and age-related diseases in Gunma Prefecture and Tokyo, Japan.

Diagnosis of metabolic syndrome was based on a modified version of the definition proposed by the American Heart Association and the U. S. National Heart, Lung, and Blood Institute [4]. In this modified version, which was also used in the West of Scotland Coronary Prevention Study [39] and the Women's Health Study [40], waist circumference is replaced by body mass index (BMI). On the basis of

the recent recognition of a need to revise BMI criteria for obesity in Japanese and other Asian populations [41], we set the cutoff point for obesity as a BMI of  $\geq 25$  kg/m<sup>2</sup>. The 2150 study subjects with metabolic syndrome thus had three or more of the following five components of this condition: (1) a BMI of  $\geq 25$  kg/m<sup>2</sup>; (2) a serum triglyceride concentration of  $\geq 1.65$  mmol/L (150 mg/dL) or drug treatment for elevated triglyceride; (3) a serum HDL-cholesterol concentration of  $<1.04$  mmol/L (40 mg/dL) for men or  $<1.30$  mmol/L (50 mg/dL) for women, or drug treatment for low HDL-cholesterol; (4) a systolic blood pressure of  $\geq 130$  mmHg, diastolic blood pressure of  $\geq 85$  mmHg, or drug treatment for hypertension; and (5) a fasting plasma glucose level of  $\geq 5.50$  mmol/L (100 mg/dL) or drug treatment for high glucose.

Estimated glomerular filtration rate (eGFR) was calculated with the use of the simplified prediction equation proposed by the Japanese Society of Nephrology and based on that described in the Modification of Diet in Renal Disease (MDRD) Study [42]:  $\text{eGFR (mL min}^{-1} 1.73 \text{ m}^{-2}) = 194 \times [\text{age (years)}]^{-0.287} \times [\text{serum creatinine (mg/dL)}]^{-1.094} \times [0.739 \text{ (if female)}]$ . The National Kidney Foundation–Kidney Disease Outcomes Quality Initiative guidelines have proposed that CKD be diagnosed if eGFR is  $<60$  mL min<sup>-1</sup> 1.73 m<sup>-2</sup> [1]. Previous observations have demonstrated nonlinear relations between GFR and the risk of adverse events, such as death, cardiovascular events, and hospitalization, with an increased risk associated with an eGFR of  $<60$  mL min<sup>-1</sup> 1.73 m<sup>-2</sup> and a markedly increased risk associated with values of  $<45$  mL min<sup>-1</sup> 1.73 m<sup>-2</sup> [43]. In addition, the rate of GFR decline was significantly higher in Japanese individuals younger than 70 years who had an initial GFR of  $<50$  mL min<sup>-1</sup> 1.73 m<sup>-2</sup> [44]. We thus adopted an eGFR of  $<50$  mL min<sup>-1</sup> 1.73 m<sup>-2</sup> as the criterion for CKD in the present study. On the basis of this criterion, 411 subjects (256 men, 155 women) were diagnosed with CKD. The control subjects comprised 1739 individuals (1050 men, 689 women) whose eGFR was  $\geq 60$  mL min<sup>-1</sup> 1.73 m<sup>-2</sup>. The study protocol complied with the Declaration of Helsinki and was approved by the Committees on the Ethics of Human Research of Mie University Graduate School of Medicine, Hirosaki University Graduate School of Medicine, Gifu International Institute of Biotechnology, Tokyo Metropolitan Institute of Gerontology, and participating hospitals. Written informed consent was obtained from each participant.

### Selection of polymorphisms

Our aim was to identify genes associated with CKD in Japanese individuals with metabolic syndrome in a case-control association study by examining the relations of one to four polymorphisms of each candidate gene to this condition. With the use of public databases, including PubMed (NCBI) and Online Mendelian Inheritance in Man (OMIM), we selected 80 candidate genes that have been characterized and suggested to be associated with CKD. On the basis of

**Table 4**Effects of genotypes and other characteristics on the prevalence of chronic kidney disease as determined by a stepwise forward selection procedure (*P*<0.05) among individuals with metabolic syndrome

Variable	P	R <sup>2</sup>
Age	<0.0001	0.0370
Diabetes mellitus	<0.0001	0.0211
Hypertension	<0.0001	0.0103
APOE (TT versus GG+GT)	0.0006	0.0056
ABCA1 (TT versus CC+CT)	0.0058	0.0036
PTGS1 (TT versus CC+CT)	0.0097	0.0032
TNF (AA+CA versus CC)	0.0117	0.0031
CPB2 (AA+GA versus GG)	0.0250	0.0024
AGTR1 (AA+GA versus GG)	0.0256	0.0023
OR13G1 (GG+AG versus AA)	0.0293	0.0022
GNB3 (TT versus CC+CT)	0.0314	0.0022
ABCA1 (GG versus AA+AG)	0.0327	0.0022



published studies or by searching PubMed and single nucleotide polymorphism (SNP) databases [dbSNP (NCBI) and Japanese SNP (JSNP)], we further selected 100 polymorphisms of these genes, most located in the promoter region or exons, that might be expected to result in changes in the function or expression of the encoded protein (Supplementary Table 2).

#### Genotyping of polymorphisms

Venous blood (7 mL) was collected into tubes containing 50 mmol/L ethylenediaminetetraacetic acid (disodium salt), and genomic DNA was isolated with a kit (Genomix; Talent, Trieste, Italy). Genotypes of the 100 polymorphisms were determined at G&G Science (Fukushima, Japan) by a method that combines the polymerase chain reaction (PCR) and sequence-specific oligonucleotide probes with suspension array technology (Luminex, Austin, TX). Primers, probes, and other PCR conditions for genotyping polymorphisms found to be related (*P* value for allele frequency of <0.05) to CKD by the chi-square test are shown in Supplementary Table 3. Detailed genotyping methodology was described previously [45].

#### Statistical analysis

Quantitative data were compared between subjects with CKD and controls by the unpaired Student's *t* test. Categorical data were compared by the chi-square test. Allele frequencies were estimated by the gene counting method, and the chi-square test was used to identify departures from Hardy-Weinberg equilibrium. In the initial screen, the genotype distribution (3×2) and allele frequencies (2×2) of each polymorphism were compared between subjects with CKD and controls by the chi-square test. Given the multiple comparisons of genotypes with CKD, the FDR was calculated from the distribution of *P* values for the allele frequencies of the 100 polymorphisms [46]. Polymorphisms with a *P* value of <0.05 for allele frequency were further examined by multivariable logistic regression analysis with adjustment for covariates. Such analysis was thus performed with CKD as a dependent variable and independent variables including age, sex (0=woman, 1=man), history of hypertension, diabetes mellitus, or hypercholesterolemia (0=no history, 1=positive history), and genotype of each polymorphism; the *P* value, odds ratio, and 95% confidence interval were calculated. Each genotype was assessed according to dominant (0=major allele homozygote, 1=heterozygote=minor allele homozygote), recessive (0=major allele homozygote=heterozygote, 1=minor allele homozygote), and additive [(0, 0)=major allele homozygote, (1, 0)=heterozygote, (0, 1)=minor allele homozygote] genetic models. Additive models included the additive 1 model (heterozygotes versus major allele homozygotes) and the additive 2 model (minor allele homozygotes versus major allele homozygotes), which were analyzed simultaneously with a single statistical model. We also performed a stepwise forward selection procedure to examine the effects of genotypes as well as of other covariates on CKD. In this analysis, each genotype was examined according to a dominant or recessive model on the basis of statistical significance in the multivariable logistic regression analysis. The *P* levels for inclusion in and exclusion from the model were 0.25 and 0.1, respectively. With the exception of the initial screen by the chi-square test (FDR<0.05), a *P* value of <0.05 was considered statistically significant. Statistical significance was examined by two-sided tests performed with JMP version 6.0 and JMP Genomics version 3.2 software (SAS Institute, Cary, NC).

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2008.11.001.

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